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Article

FTIR Screening to Elucidate Compositional Differences in Maize Recombinant Inbred Lines with Contrasting Saccharification Efficiency Yields

Ana López-Malvar ^{1,2,*} , Rogelio Santiago ¹, Rosa Ana Malvar ² , Daniel Martín ³ , Inês Pereira dos Santos ³, Luís A. E. Batista de Carvalho ³ , Laura Faas ⁴, Leonardo D. Gómez ⁴  and Ricardo M. F. da Costa ^{3,5}

¹ Facultad de Biología, Departamento de Biología Vegetal y Ciencias del Suelo, Universidad de Vigo, As Lagoas Marcosende, 36310 Vigo, Spain; rsantiago@uvigo.es

² Misión Biológica de Galicia (CSIC), Pazo de Salcedo, Carballeira 8, 36143 Pontevedra, Spain; rmalvar@mbg.csic.es

³ Molecular Physical-Chemistry R&D Unit, Department of Chemistry, University of Coimbra, Rua Larga, 3004-535 Coimbra, Portugal; dmfernandez@uc.pt (D.M.); ips@uc.pt (I.P.d.S.); labc@ci.uc.pt (L.A.E.B.d.C.); rmfdcosta@uc.pt (R.M.F.d.C.)

⁴ Centre for Novel Agricultural Products, Department of Biology, CNAP, University of York, York YO10 5DD, UK; laura.faas@york.ac.uk (L.F.); leonardo.gomez@york.ac.uk (L.D.G.)

⁵ Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal

* Correspondence: alopezmalvar@mbg.csic.es



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Abstract: With a high potential to generate biomass, maize stover arises as an outstanding feedstock for biofuel production. Maize stover presents the added advantage of being a multiple exploitation of the crop as a source of food, feed, and energy. In this study, contrasting groups of recombinant inbred lines (RILs) from a maize multiparent advanced generation intercross (MAGIC) population that showed variability for saccharification efficiency were screened by FTIR-ATR spectroscopy to explore compositional differences between high and low saccharification yielders. High and low saccharification efficiency groups differed in cell wall compositional features: high saccharification RILs displayed higher proportions of S subunits, aromatic compounds, and hemicellulose as opposed to low saccharification efficiency groups in which FTIR predicted higher proportions of lignin, more precisely lignin being richer in subunits G, and greater proportions of crystalline cellulose and acetyl methyl esters. The application of FTIR-ATR spectroscopy in this material allowed us to obtain a rapid and broad vision of cell wall compositional features in contrasting groups of saccharification efficiency. These results helped us to deepen our knowledge into the relationship between cell wall composition and biorefining potential; they also allowed us to establish new targets for future research regarding lignocellulosic bioconversion.

Keywords: FTIR-ATR; saccharification efficiency; cell wall; MAGIC

1. Introduction

Besides its uses as food and feed, maize stover after ear removal can have biorefinery applications. Cellulosic ethanol derived from fast growing C4 crops has become one of the preferred alternatives to fossil fuels due to their high biomass yields, broad geographic adaptation, carbon sequestration, and nutrient utilization [1,2]. Maize has a high biomass yield potential (5.2 tons of dry matter/ha; dry biomass yield) and has been proposed as an outstanding model for biofuel production [3,4].

Lignocellulosic feedstock, such as maize stover, is highly abundant and readily available as substrates for second-generation biofuel production [1,5]. It is typically composed of 39.4% cellulose, 33.1% hemicelluloses, and 14.9% lignin [6]. Lignin concentration and composition present variations among taxa and tissue types [7]. In mature stalk, G and S

lignin units are prevalent compared to H units with average ratios that are nearly equal to 35% and 4%, respectively [8].

The conversion of lignocellulosic biomass to ethanol is a three-step process, namely (i) a pretreatment stage, followed by (ii) a hydrolytic degradation of carbohydrates to the constituent sugar monomers (saccharification), and (iii) a final fermentation of the free sugars to ethanol [9]. The main obstacle for the biomass fermentation process is cell wall recalcitrance, defined as cell wall resistance to degradation by microbial cellulolytic complex. Recalcitrance increases the energy requirements, the cost, and complexity of bio refinery operations and reduce the recovery of biomass carbon into desired products [10]. Therefore, the reduction of cell wall recalcitrance by overcoming chemical and structural properties of the cell wall is expected to improve saccharification efficiency and increase the sugars that could be fermented [11].

The saccharification process is dependent on the composition and architecture of the cell wall. One of the key traits for the processing of plant biomass to produce biofuels and biomaterials is cell wall quality [12,13]. Efforts to reduce the inherent recalcitrance of bioenergy feedstock have focused on understanding how variations in lignin content, composition, and structure can alter the bioconversion process. Lignin reduces the efficacy of enzymatic saccharification processes by adsorbing and nonproductively binding to hydrolytic enzymes [14,15] and by physically shielding cellulose microfibrils from enzymatic attack [12]. The variations in lignin monomeric composition (such as the way lignin monomers are linked or the increase in S-lignin proportions) have proven useful for enhancing extraction efficiency. A key determinant of cell wall architecture are crosslinks between polysaccharides and lignin via hydroxycinnamates [12]. These bonds restrict the accessibility of exogenous enzymes to the cell wall polysaccharides, hindering biomass hydrolysis [6,13]. Finally, the degree of polymerization of the cellulose and its crystallinity index have also been associated to reductions in biomass recalcitrance [15–17].

López-Malvar et al. [18] evaluated a subset of recombinant inbred lines (RILs) derived from a multiparent advanced generation intercross (MAGIC) population, and they found variability for saccharification efficiency in samples of maize stover after alkaline pretreatment. From that RIL subset, we selected the lines showing the highest and lowest saccharification efficiency, and in the current study we screen them by Fourier-transform infrared spectroscopy in attenuated total reflectance mode (FTIR-ATR) which, in contrast to analytical/biochemical methods, is a rapid, noninvasive and powerful high-throughput tool to study the cell wall. This technique has been extensively used for the study of the cell wall components, crosslinking, and carbohydrate constituents and their organization [19,20].

The aim of this study is to take advantage of this spectroscopy technique applied to contrasting saccharification efficiency groups in order to elucidate differences in the cell wall composition and architecture of high and low sugar-yielding RILs. In addition, as far as we know, this is first time that FTIR-Principal Component Analysis is used on maize RILs to correlate cell wall traits with saccharification. It is expected that the results obtained in this research will show the relationship between the composition of the wall and the cellulose bioconversion process in stover samples from highly variable RILs, which could reveal key aspects to optimize the conversion of not only lignocellulosic biomass biorefinery but also other promising bioproducts such as biogas via fermentation with anaerobic bacteria or nonbiofuel-related products derived from fermentation of lignocellulose derived sugars.

2. Material and Methods

A subset of 408 RILs and the eight founders of a MAGIC population developed by the Maize Genetics and Breeding group at Misión Biológica de Galicia-CSIC [21,22] were evaluated in single augmented design with 10 blocks in Pontevedra for two years (2016, 2017). Saccharification efficiency was considered as the amount of released sugars ($\text{nmol mg}^{-1} \text{ material}^{-1} \text{ h}^{-1}$) after alkaline pretreatment: 0.5 M NaOH at 90 °C for 30 min, washed four times with 500 μL sodium acetate buffer, and subjected to enzymatic digestion

(Celluclast CTec2, 7FPU/g) at 50 °C for 8 h [23]. For detailed material and methods determination, see López-Malvar et al. [18]

2.1. Fourier-Transform Infrared Spectroscopy

Attenuated total reflectance Fourier transform mid-infrared (FTIR-ATR) spectroscopy was performed on all samples included in this study, as reported elsewhere [20,24]. Duplicate spectra were collected in the range 4000–400 cm^{-1} using a Bruker Optics Vertex 70 FTIR spectrometer purged by CO_2 -free dry air and equipped with a Bruker Platinum ATR single reflection diamond accessory. A Ge on KBr substrate beamsplitter and a liquid nitrogen-cooled wide-band mercury cadmium telluride (MCT) detector were used. Spectra were averaged over 32 scans at a resolution of 4 cm^{-1} , and the 3-term Blackman–Harris apodization function was applied. The Bruker Opus 8.1 software was also used for (i) removing eventual H_2O and CO_2 contributions and (ii) spectral smoothing using the Savitzky–Golay algorithm. Absorbance spectra were further preprocessed using in-house built functions in MATLAB (v. R2014b; MathWorks, Natick, MA, USA). Full spectra, or fingerprint region spectra (1800–800 cm^{-1}), were averaged (per replicate), vector normalized to unit length, and the baseline was removed according to the automatic weighted least squares algorithm (polynomial order = 2) prior to statistical analysis, using the Eigenvector PLS Toolbox (v. 7.9; Eigenvector Research, Wenatchee, WA, USA). For the t-tests on spectral data to unveil the underlying chemometric relationships between FTIR-ATR spectra, an R-based data analysis platform was used [Chong 2018-<https://doi.org/10.1093/nar/gky310> (accessed on 1 August 2020)].

2.2. Statistical Analysis

An analyses of variance was done for saccharification efficiency according to the SAS mixed-model procedure (PROC MIXED) of the SAS program, version 9.4 (SAS Institute 2015). Inbred lines were considered as the fixed effects, while years, replication within years, and lines \times year were considered random effects. The comparison of means among inbred lines was carried out using Fisher's protected least significant difference (LSD). In order to classify the RILs into high- and low-saccharification groups, data from each year were considered separately since we observed the RIL \times year interaction. From each year we selected a total of 60 RILs according to its saccharification efficiency (30 high yielders, 30 low yielders). High and low groups differed significantly in their saccharification efficiency ($\text{LSD} < 0.01$).

3. Results and Discussion

A subset of 408 RILs from a maize MAGIC population were evaluated for saccharification efficiency and analyzed by FTIR-ATR spectroscopy in field experiments over two years.

RILs differed significantly for saccharification efficiency (Supplementary Table S1). A total of 778 samples of maize ground biomass were analyzed in duplicate by FTIR-ATR spectroscopy, for a total of 1556 data points. A preliminary principal component analysis (PCA) model was calculated, including all the data. No natural data clusters were detected, suggesting that the samples were not significantly distinct from each other.

We observed a large influence of the year for saccharification efficiency, but we did not observe a year \times genotype interaction, indicating that even though saccharification efficiency value ranges are different for each year, RILs that in one year present high saccharification efficiency would also present high values in the other year (data not shown).

Using the information from the best linear unbiased estimator (BLUEs) from each year, the 30 lowest and the 30 highest lines according to the saccharification yields were selected to form two extreme groups (Table 1). A t-test ($p \leq 0.001$) was performed for each variable (wavenumber) to compare between high- and low-saccharification groups. For the 2016 assay, 208 variables were significantly different, whereas for 2017, 121 variables were significantly different. Two PCA models (2016 and 2017) were calculated based

on these highly significantly different variables. In both cases, separation between high- and low-saccharification groups occurred along PC1 (Figure 1). Subsequently, based on PC1 loadings, tentative compositional attributions were made (Table 2) in order for us to understand the underlying relationships between the groups of spectra.

Table 1. Saccharification efficiency data, means, and range for two years (2016, 2017) in selected RILs from a MAGIC population classified in high and low saccharification yielders.

| 2016 | | 2017 | |
|-------------|--|-------------|--|
| RILs | Saccharification (nmol mg ⁻¹ material ⁻¹ h ⁻¹) | RILs | Saccharification (nmol mg ⁻¹ material ⁻¹ h ⁻¹) |
| Low | | | |
| EPS21LR-415 | 70.354 | EPS21LR-267 | 138.409 |
| EPS21LR-494 | 73.340 | EPS21LR-280 | 140.569 |
| EPS21LR-629 | 77.574 | EPS21LR-560 | 142.657 |
| EPS21LR-711 | 78.112 | EPS21LR-347 | 142.719 |
| EPS21LR-289 | 78.183 | EPS21LR-283 | 142.979 |
| EPS21LR-748 | 78.336 | EPS21LR-409 | 143.652 |
| EPS21LR-472 | 79.001 | EPS21LR-597 | 143.789 |
| EPS21LR-643 | 79.213 | EPS21LR-703 | 143.864 |
| EPS21LR-414 | 79.443 | EPS21LR-260 | 144.628 |
| EPS21LR-526 | 79.588 | EPS21LR-698 | 144.732 |
| EPS21LR-316 | 80.571 | EPS21LR-522 | 148.238 |
| EPS21LR-353 | 80.747 | EPS21LR-670 | 149.194 |
| EPS21LR-547 | 81.101 | EPS21LR-614 | 149.238 |
| EPS21LR-317 | 81.409 | EPS21LR-348 | 150.578 |
| EPS21LR-698 | 81.641 | EPS21LR-398 | 150.623 |
| EPS21LR-753 | 82.205 | EPS21LR-284 | 151.153 |
| EPS21LR-675 | 82.983 | EPS21LR-578 | 152.176 |
| EPS21LR-416 | 82.984 | EPS21LR-619 | 152.182 |
| EPS21LR-524 | 83.346 | EPS21LR-514 | 152.870 |
| EPS21LR-285 | 83.747 | EPS21LR-617 | 152.978 |
| EPS21LR-750 | 83.753 | EPS21LR-427 | 153.046 |
| EPS21LR-522 | 83.909 | EPS21LR-414 | 153.132 |
| EPS21LR-426 | 83.936 | EPS21LR-462 | 153.548 |
| EPS21LR-284 | 83.998 | EPS21LR-669 | 153.581 |
| EPS21LR-759 | 84.292 | EPS21LR-253 | 154.624 |
| EPS21LR-251 | 84.670 | EPS21LR-740 | 155.013 |
| EPS21LR-378 | 84.768 | EPS21LR-361 | 155.073 |
| EPS21LR-677 | 85.155 | EPS21LR-682 | 155.156 |
| EPS21LR-257 | 85.341 | EPS21LR-411 | 155.316 |
| EPS21LR-478 | 85.468 | EPS21LR-276 | 155.693 |
| Means | 81.306 | Means | 149.380 |
| Range | 70.354–85.468 | Range | 138.409–155.693 |

Table 1. Cont.

| 2016 | | 2017 | |
|-------------|--|-------------|--|
| RILs | Saccharification (nmol mg ⁻¹ material ⁻¹ h ⁻¹) | RILs | Saccharification (nmol mg ⁻¹ material ⁻¹ h ⁻¹) |
| High | | | |
| EPS21LR-749 | 114.589 | EPS21LR-539 | 196.892 |
| EPS21LR-623 | 114.671 | EPS21LR-304 | 196.911 |
| EPS21LR-663 | 114.899 | EPS21LR-325 | 196.961 |
| EPS21LR-395 | 115.104 | EPS21LR-483 | 197.079 |
| EPS21LR-259 | 115.128 | EPS21LR-753 | 197.152 |
| EPS21LR-261 | 115.189 | EPS21LR-646 | 197.893 |
| EPS21LR-243 | 115.207 | EPS21LR-598 | 198.361 |
| EPS21LR-489 | 115.817 | EPS21LR-451 | 199.220 |
| EPS21LR-337 | 116.051 | EPS21LR-396 | 200.177 |
| EPS21LR-473 | 116.829 | EPS21LR-442 | 200.615 |
| EPS21LR-657 | 117.058 | EPS21LR-655 | 201.290 |
| EPS21LR-709 | 117.321 | EPS21LR-381 | 201.830 |
| EPS21LR-325 | 117.476 | EPS21LR-515 | 202.263 |
| EPS21LR-653 | 117.506 | EPS21LR-672 | 203.171 |
| EPS21LR-720 | 117.547 | EPS21LR-482 | 203.753 |
| EPS21LR-503 | 117.935 | EPS21LR-259 | 203.789 |
| EPS21LR-405 | 118.170 | EPS21LR-487 | 204.326 |
| EPS21LR-695 | 118.372 | EPS21LR-412 | 204.728 |
| EPS21LR-512 | 118.901 | EPS21LR-343 | 204.963 |
| EPS21LR-726 | 119.333 | EPS21LR-517 | 205.161 |
| EPS21LR-560 | 119.530 | EPS21LR-741 | 205.377 |
| EPS21LR-514 | 120.591 | EPS21LR-547 | 205.750 |
| EPS21LR-741 | 120.596 | EPS21LR-395 | 205.809 |
| EPS21LR-733 | 120.959 | EPS21LR-508 | 206.084 |
| EPS21LR-668 | 121.101 | EPS21LR-248 | 208.187 |
| EPS21LR-364 | 121.287 | EPS21LR-723 | 209.809 |
| EPS21LR-246 | 121.350 | EPS21LR-694 | 211.761 |
| EPS21LR-584 | 123.927 | EPS21LR-594 | 212.177 |
| EPS21LR-511 | 125.722 | EPS21LR-679 | 218.327 |
| EPS21LR-743 | 127.652 | EPS21LR-502 | 218.694 |
| Means | 118.527 | Means | 203.950 |
| Range | 114.589–127.652 | Range | 198.892–218.694 |

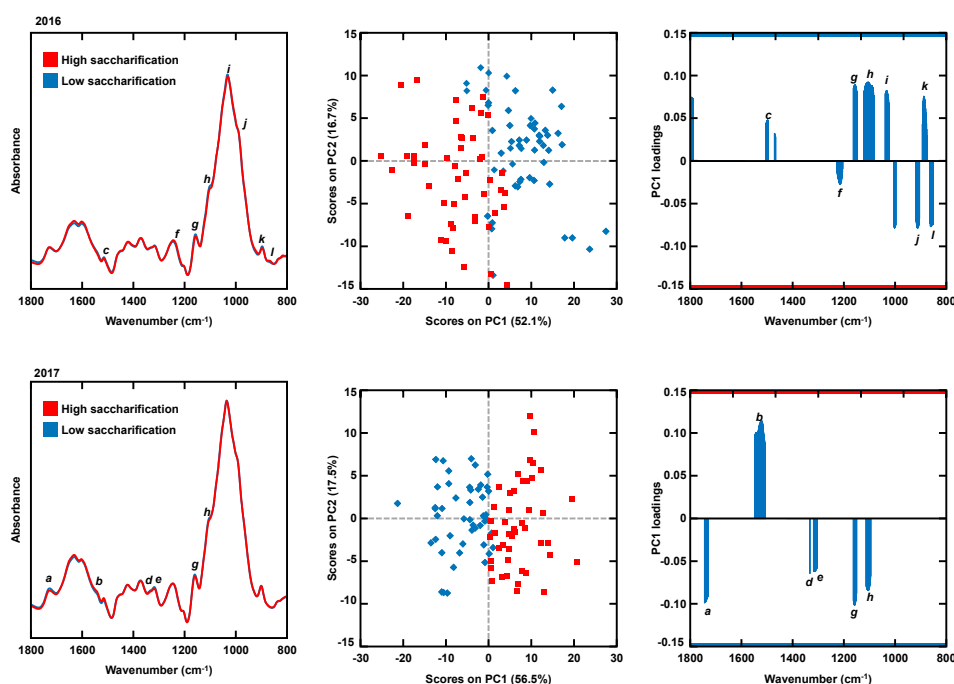


Figure 1. Mean FTIR-ATR spectra of maize lignocellulosic biomass from high and low saccharification yield groups, harvested in 2016 and 2017, in the range 1800–800 cm^{-1} . Score plot of the PCA model calculated based on highly significantly different variables ($p \leq 0.001$), as calculated by t-tests (208 variables for 2016 and 121 variables for 2017). The PC loadings plots indicate spectral regions (a–l) that are positively or negatively correlated to PC1, along which group separation occurred. Table 2 shows the wavenumbers corresponding to each region.

In the 2016 model, the high-saccharification group was clustered on the negative portion of PC1, which is negatively correlated with spectral bands ascribed to the molecular features associated to syringyl (S-lignin) units (f), aromatic compounds (j), and to hemicelluloses (l) (Table 2, Figure 1) [24–29]. By contrast, PC1 is positively correlated with spectral regions ascribed to lignin structural features, namely to guaiacyl (G-lignin) units (c, h, and i), and to cellulose structural features, e.g., to crystalline cellulose (g and k) (Table 2, Figure 1) [26,28,30–36]. Given that the low-saccharification group is mostly clustered on the positive side of PC1, samples classified as low yielders were predicted to have higher proportions of these compositional and structural cell wall traits.

In the 2017 model, the high-saccharification group is clustered on positive regions of PC1, which is negatively correlated to the following structural features: acetyl and methyl esters (a), lignin (h), and cellulose structural features (d and e), such as its degree of crystallinity (g) (Table 2, Figure 1) [30–45].

Table 2. Assignment of relevant FTIR-ATR absorption bands characteristic of maize cell wall biomass.

| PC1 Loading Spectral Region | Wavenumber (cm^{-1}) | Reference | Assignment | Biomass Constituent |
|-----------------------------|---------------------------------|---------------|--|--------------------------|
| a | 1740 | [37,40,43,44] | C=O stretching | Acetyl and methyl esters |
| | 1735 | | | |
| | 1734 | | | |
| b | 1550 | [29,30,37] | Amide II (N-H deformation and stretching contribution from C-N stretching) | Proteins |

Table 2. Cont.

| PC1 Loading Spectral Region | Wavenumber (cm ⁻¹) | Reference | Assignment | Biomass Constituent |
|-----------------------------|--------------------------------|---------------|---|--|
| <i>c</i> | 1504 1500 | [26,28] | Aromatic skeletal vibrations in guaiacyl rings | Lignin |
| <i>d</i> | 1336 | [38,41] | C-H in plane deformation | Cellulose |
| <i>e</i> | 1328 1311 1318 1311 | [38,39,43–46] | OH in-plane bending CH ₂ wagging CH in plane scissoring | Cellulose |
| <i>f</i> | 1224 1219 1220 | [27,28] | C–O stretch in syringyl rings | Lignin |
| <i>g</i> | 1160 1161 1163 1157 | [30–35] | C–O–C asymmetric stretching | Crystalline cellulose; associated to modifications cellulose-I > cellulose-II; linked to celluloses crystallinity features |
| <i>h</i> | 1116 | [31,36] | Aromatic C–H deformation | Lignin |
| <i>i</i> | 1035 1041 1040 | [24,29,47] | Aromatic C–H in plane deformation, G > S; plus C–O deformation in primary alcohols; plus C=O stretch (unconjugated) | Lignin |
| <i>j</i> | 915–925 | [24,29] | C-H out-of-plane; aromatic compounds | Aromatic compounds |
| <i>k</i> | 898 | [35] | C–O–C stretching | Cellulose |
| <i>l</i> | 872 875 | [25] | Glycosidic linkage in hemicelluloses | Hemicellulose |

Biomass hydrolysis is a key factor in lignocellulosic deconstruction during biofuel production. Among other cell wall components such as ester linked hydroxycinnamates or arabinose/xylose ratio, lignin has been pointed out as one of the most important polymer in the determination of biomass recalcitrance, not only because it makes the biomass resistant to digestion, but also because lignin fractions adsorb enzymes, reducing their access to the polysaccharides [47–49]. Reductions in lignin content are positively correlated with increases of cell wall hydrolysis efficiency [50,51]. This is supported by the results obtained in this study, as higher saccharification yields in 2017 are related with FTIR predictions of decreasing concentrations of lignin. Besides total lignin content, its composition and the manner in which it binds holocellulose within the cell wall matrix is often seen as a feature of cell wall recalcitrance to enzymatic deconstruction [47]. Modifications in the phenylpropanoid pathway, through enzymes directly changing lignin content and monolignol composition, were associated with increases in saccharification efficiency in several crops [52]. For example, transgenic switchgrass lines with reduced cinnamyl alcohol dehydrogenase (CAD) levels and consequently reduced lignin content and altered lignin composition showed improved sugar release [53]. Similarly, Fornalé et al. [54] studied the effect of CAD downregulation on lignin S/G ratio, among other cell wall components, through a transgenic approach in maize. They observed a decrease in the syringyl-to-guaiacyl (S/G) ratio by both a reduction in the syringyl (S) subunits and an increase of the guaiacyl (G) and p-hydroxyphenyl (H) subunits. Moreover, they observed that transgenic plants produced 8% more cellulosic bioethanol than the wild type. The results obtained in the present study support the idea that lignin monomeric composition influences the saccharification efficiency of the feedstock. High saccharification yields in this MAGIC

population correlate with lignin subunits, i.e., lower proportions of G subunits in contrast to higher proportions of S subunits, which are known to promote saccharification efficiency. S units can only form β -O-4- (β -aryl ether) inter-unit linkages, which are easily cleaved onto. By contrast, G units have the availability of the C5 position for coupling. Lignin composed mainly of G units contain more resistant (β -5, 5-5, and 4-O-5) linkages than lignins incorporating S units [55,56].

However, variation in cell wall recalcitrance is not only due to lignin content and composition. In concordance with the results obtained, cell wall aromatic compounds, such as ferulic acid, could be positively correlated to saccharification in grass species [57]. To support this, FTIR predicted that RILs classified in the low saccharification efficiency group would present lower proportions of aromatic compounds.

On the other hand, lignocellulosic polysaccharides, mainly cellulose (40%), serve as the main substrate for the fermentation of cell wall sugars in ethanol. In this way, cell walls richer in cellulose have more sugars to potentially be fermented. The low-saccharification groups in 2017 were predicted to present lower proportions of cellulose structural features, which are detrimental for biomass deconstruction. The results obtained in 2016 show that low-saccharification groups presented higher cellulose crystallinity, indicating that crystallinity limit enzymatic degradability. Cellulose is composed of linear chains of D-glucopyranose residues linked by β -(1-4) glucosidic bonds that result in the formation of glucose dimers between adjacent chains that form a flat structure called cellobiose that is repeated. The structure of the cellulose chains allows for the formation of intermolecular hydrogen bridges. This results in a stable crystalline structure that provides cellulose mechanical strength and stability, contrasting with amorphous less organized regions [58]. The crystalline structure limits the penetration of water molecules and is highly resistant to chemical and biological hydrolysis to form fermentable sugars [10,57,59,60], thus decreasing saccharification efficiency and hydrolysis yield potential.

The interaction between cellulose and other cell wall features, such as the pattern of xylan acetylation, also influences saccharification since a uniform pattern of xylan substitution is crucial within plant cells interactions with cellulose [61]. In grass cell walls, most of the acetylation occurs in arabinoxylans, which modifies the interaction with cellulose and lignin [62]. The role of acetylation in biomass recalcitrance was demonstrated in other Poales species, such as in *Miscanthus* spp. [57]. In this study, we suggest that acetate could cause steric hindrance of hydrolytic enzymes, thus inhibiting both saccharification and fermentation. Pawar et al. [63] found that aspen plants with reduced xylan acetylation showed 25% higher glucose saccharification yield compared with wild types. They proposed that de-acetylating xylan increases susceptibility to hydrolytic enzymes during saccharification as well as promoting changes in the cell wall architecture that increase the extractability of lignin and xylan.

4. Conclusions

This study is one of the first research that used FTIR-PCA on maize RILs to correlate cell wall traits with saccharification. High- and low-saccharification groups differed in its cell wall spectral features, and FTIR spectroscopy in this material revealed proper cell wall compositional features for saccharification efficiency. The results presented here help us to understand the relationship between maize cell wall composition and its potential for biofuel production. This can allow for establishing new targets for future research and breeding targets to tailor biorefinery feedstock, for which this maize MAGIC population is a useful genetic tool that presents great advantages, such as high genetic diversity and rapid linkage disequilibrium decay.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/agronomy11061130/s1>, Table S1. Average, range values and BLUEs for saccharification efficiency in RILs of the MAGIC population.

Author Contributions: A.L.-M., R.M.F.d.C., R.A.M., and R.S. conceived and design the study. R.A.M., R.S., and A.L.-M. participated in its design and carried out the field trial and participated in sample collection; A.L.-M. wrote the manuscript; L.F. and L.D.G. performed saccharification efficiency analysis and contributed to the discussion of the manuscript; A.L.-M. and R.A.M. participated in saccharification efficiency data analysis; R.M.F.d.C. performed FTIR-ATR and statistical analysis; D.M. participated in spectral interpretation of FTIR-ATR; I.P.d.S. participated in statistical analysis of FTIR-ATR; L.A.E.B.d.C. is responsible for the FTIR equipment, and contributed significantly to the discussion of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: Authors declare that they have no conflict of interest.

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